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Structures of Botulinum Neurotoxin, Its Functional Domains, and Perspectives on the Crystalline Type A Toxin

Bibhuti R. DasGupta
University of Wisconsin, Madison, Wisconsin

INTRODUCTION

It was 1895. In the village of Ellezelles, Belgium, 34 members of a music club had eaten some raw salted ham after performing at a funeral. Within the next 20 to 36 hours most of the musicians developed a neuroparalytic syndrome—three died, and 10 others nearly died. Professor E. Van Ermengem isolated the culprit from the food and the victims of food poisoning and named it *Bacillus botulinus*; the anaerobic bacterium was later named *Clostridium botulinum* (1,2). The neurotoxic substance produced by the bacterium became known as botulinum toxin, a protein, and gained notoriety as the most poisonous poison (3). In the New World, 84 years after the discovery of the agent of deadly food poisoning, Dr. Allen B. Scott of San Francisco first reported how to use the most poisonous poison for therapeutic purposes in humans (4,5). Because of this pioneering breakthrough, lauded by Carl Lamanna (6) as "the most imaginative, technically brilliant, and courageous of such applied research," today more people in the United States are exposed to botulinum toxin through deliberate injection by clinicians (7) than by unknowingly ingesting it as a food poison (8).

The proverbial beating of swords into ploughshares was achieved with a body of knowledge mostly accumulated from the pharmacological studies of the toxin (primarily serotype A, see below), which in 1946 became available as a crystallizable preparation (9–11), and with sparse knowledge about the biochemistry of the neurotoxin (NT) (12) and its mechanism of action (13). Another remarkable aspect of this development is the low-grade purity of the NT preparation that was used in the early pharmacological studies (12–15), as well as in the pioneering therapeutic application, and is still being widely used.

Now we know that four primary sequential events lead to the manifestation of the well-known neurotoxicity of botulinum NT: (1) the NT binds to the cholinergic membrane at

the neuromuscular junctions, (2) the bound protein reaches inside the nerve endings through the endocytotic/lysosomal vesicle pathway, (3) a segment of the NT penetrates through the endosomal membrane into the cytosol, and then (4) this segment, acting as an enzyme, disrupts the neurotransmitter secretory machinery, resulting in blockage of acetylcholine release and consequent flaccid paralysis. The entire structure of the NT may be regarded in a simple model as a combination of three structural segments designated A-C-B, each with a functional role and joined linearly in that order. Segment B recognizes the NT-specific receptors and allows the NT to bind to the target cells. Segment C forms channels in the endosomal membrane and facilitates penetration of segment A (or A plus segments of C and B) into the cytosol. The enzymatically active segment A causes intracellular injury.

This chapter deals with two primary topics. First, the structural features of the NT and its three segments that now appear directly relevant to the NT's mechanism of action are discussed. A comprehensive review in this area is not presented. The second topic is a critique of the crystalline toxin type A, which contains no more than 20% by weight of the neurotoxin protein, the other 80% of the material being nonneurotoxic macromolecules (16,17) that are yet to be characterized; nevertheless, this is the preparation used clinically (18,19).

THE NEUROTOXIN

Sources of the Neurotoxin

The NT produced by the ubiquitous bacterium *C. botulinum* is found as seven antigenically distinguishable serotypes A, B, C, D, E, F, and G. Certain strains of *C. baratii* and *C. butyricum* have been identified within the past few years that produce NTs similar to classical botulinum NT serotypes F and E, respectively (2,20,21). A minor antigenic relatedness between types E and F has been known (22); with the advent of monoclonal antibodies a common epitope has been detected among types B, C, D, and E (23). One strain of the bacterium produces one serotype, but there may be exceptions to this (20,22). Serotype C at one time had two designations, C1 and C2. The C2 toxin, which ADP-ribosylates nonmuscle actin, is not the NT (2).

Structure of the Neurotoxin

Primary (Covalent) Structure

The neurotoxic protein is synthesized as a single-chain protein (mol. wt. ~150,000). Protease(s) endogenous to the bacterium cleave the single-chain protein within a narrow region inside a disulfide loop located about one-third of the way from the N-terminal to the C-terminal (Fig. 1a). This cleavage is called nicking. The proteolytically processed product—the dichain NT—now contains a light (L) chain (mol. wt. ~50,000) and a heavy (H) chain (mol. wt. ~100,000) that remain linked by an inter-chain disulfide or disulfides and noncovalent bonds. In the absence of the endogenous proteolytic enzymes the single-chain NT remains as such and after isolation from the bacterial culture can be nicked by the exogenous proteases (such as trypsin) into the dichain protein. The proteolytically processed dichain NT is more potent than the single-chain NT. The higher potency is evident whether the assay is mouse lethality, paralysis of neuromuscular junction preparations, or blockage of neurotransmitter release (24–27). This enhancement of biological activity is referred to as activation (28). The type A NT isolated from a 96-hr

incubated bacterial culture is found in the dichain form and fully activated (i.e., treatment with trypsin does not activate it further). The type E NT isolated from a 96-hr incubated culture is a single-chain protein, and following controlled trypsinization (nicking) the dichain NT exhibits more than 100-fold activation (12,28). The proteolytic processing at the nicking region involves cleavage of more than a single peptide bond; the result is excision of several amino acid residues. In the cases of type A and type E at least four residues (Gly-Tyr-Asn-Lys) and three residues (Gly-Ile-Arg), respectively, are removed (29,30), as depicted in Fig. 1a.

The complete amino acid sequences of NT serotypes A–F have been deduced on the basis of the corresponding nucleotide sequences (31,32 and references in Ref. 31). The predicted amino acid sequences indicate that the single-chain NTs are made of 1295 to 1251 amino acid residues; they are schematically represented in Fig. 2a. Among these six serotypes type E is the shortest (1251 residues) and type A is the longest (1295 residues). During proteolytic processing of the single-chain NT at the nicking region, additional cleavage at the NT's N-terminal (and hence excision of small peptides) does not occur. Direct amino acid sequence determinations of the NT types A, B, and E have demonstrated that their original N-terminals remain intact. Whether the original C-terminals of the single-chain NT remain in the proteolytically processed NT is yet to be determined.

Alignment of the amino acid sequences (not presented here, but see Refs. 31 and 32 for details) show an overall low homology (~50%), although several short stretches of varying lengths are homologous. This is understandable, because too much homology in sequence would endow the NT serotypes with common antigenic epitopes. Since these proteins do not show serological cross-reactivity (except as mentioned before), their primary and secondary structures are likely to be more dissimilar than similar. The similarity among them is likely to be just enough to conserve the structures required for similar functional properties such as receptor binding, channel formation, and intracellu-

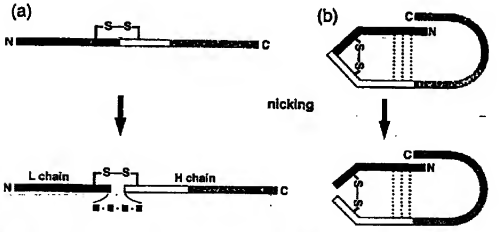

Figure 1 Proteolysis (nicking) converts the single-chain neurotoxin (NT) to dichain NT. Structure of the NT is represented in the straight line (panel a) and folded (panel b) configurations. Cleavages of more than one peptide bond during nicking are depicted by release of four amino acid residues. The light (L) and heavy (H) chains (mol. wt. ~50,000 and ~100,000, respectively) of the dichain (nicked) NT remain linked by a disulfide bond and noncovalent bonds (dotted lines between the L and H chain, see panel b). The two halves of the H chain, the N-terminal and C-terminal halves, are identified as the white and shaded segments, respectively.

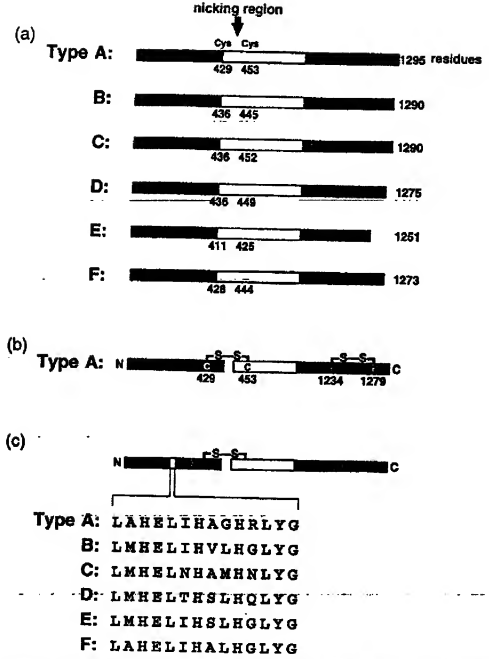

Figure 2 Covalent structures of the neurotoxin (NT) serotypes and a few of their important homologous segments. (a) Total number of amino acid residues, deduced from nucleotide sequences (31,32) present in the single chain NTs before proteolytic processing; also identified are the Cys residues that probably form the interchain disulfide in the dichain NTs. (b) Dichain type A NT and its Cys residues that have been deduced to form inter- and intrachain disulfides. (c) Homologous segment of the L chains of the NTs, containing the sequence His-Glu-Axx-Axx-His-Axx-Axx-His, and the zinc-binding motif.

lar inhibitory action (note below that even some of these properties of the NT serotypes are not identical).

Two Cys residues, the locations of which are conserved among all serotypes and indicated by the residue numbers (e.g., 429 and 453 in type A), flank the nicking region (Fig. 2a). These two Cys residues in the single-chain protein probably form the intrachain disulfide. The length of this disulfide bridge varies from 23 residues (in type A) to 8 residues (in type B). This intrachain disulfide, following nicking of the single-chain NT to the dichain NT, presumably acts as the interchain disulfide between the L and H chain. Evidence for this is inferential; direct chemical proof is yet to come. An intrachain disulfide near the C-terminal of types A and B has been also deduced (see reference in Ref. 33). In the case of Type A, Cys 1234 and Cys 1279 form this intrachain disulfide, as shown in Fig. 2b (33). Type B and E NTs have Cys residues in equivalent locations (31).

Roles of Cys Residues in Biological Activity

Long before the exact number of Cys residues present in type A NT became known (from its amino acid composition and sequences), their importance in the protein's neurotoxicity as free –SH groups and participants of disulfide bond(s) was of obvious interest. On the basis of the simple mouse lethality assay, the free -SH groups were found not essential for toxicity (34), but the integrity of the disulfide bond(s) was found essential (35). These early conclusions have recently been confirmed and refined in the context of the distinct steps of the NT's mechanism of action and the activities of the three functional domains of the NT. It is now known (1) that the productive binding of type A NT with the receptors does not depend on the disulfide bonds (inter- and intrachain) and free -SH groups; (2) that internalization (of the L chain) depends on the integrity of one or both disulfides (experiments could not distinguish between the inter- and intrachains, since selective reductive cleavage of one was not possible); (3) that the L chain's intracellular inhibitory activity does not require a free -SH group (because the L chain was active following alkylation of the free -SH) (36); and (4) that removal of the 32 C-terminal residues of the L chain (which includes Cys 429) does not diminish its intracellular activity (37). Points 2 and 4 together indicate (1) that disulfide bond(s) linking L and H chains, and the Cys 429 on the L chain (see Fig. 2b), have no role in the actual intracellular inhibitory action of the L chain, and (2) that the roles of the interchain disulfide (between Cys 429 and Cys 453) and the participant Cys 429 are to maintain a covalent linkage between the L chain and H chain, after nicking of the single-chain protein—otherwise the L chain, being easily separable from the H chain, might not reach the target cell interior (see below, Fig. 4). These deductions are consistent with the observation that the dichain NT presented to the cytosol of permeabilized cells, bypassing the steps of receptor binding and internalization (by endosomes), shows higher intracellular inhibitory activity after reduction of the interchain disulfide between the L and H chains (27); reduction presumably allows them to separate.

The Zinc-Binding Site of the Neurotoxin

A segment around the midsection of the L chain of all six NT serotypes sequences (Fig. 2c) contains the sequence His-Glu-Axx-Axx-His, which is the zinc-binding motif of Zn endopeptidases. The three proteases thermolysin, *Bacillus cereus* neutral protease, and *Pseudomonas aeruginosa* elastase contain Zn, and their three-dimensional structures, determined by x-ray crystallography, indicate that in each case an atom of Zn^{2+} is bound by a tetrahedral coordination with the two His of the motif His-Glu-Axx-Axx-His, while

the Glu residue binds a water molecule acting as the third Zn^{2+} ligand. The fourth ligand is another Glu residue (see Ref. 38 for further references). On the basis of this clue, atomic absorption analysis of type A, B, and E NTs were made; all three NTs contain one Zn atom per molecule of NT (mol. wt. ~150,000). Measurements of Co, Cu, Fe, Mn, and Ni were also made, and none was detected. Additional experiments demonstrated that this peptide segment binds Zn and that two His residues are involved in Zn coordination (38). The possibility that the NTs could be Zn endoproteases was tested, and proteolytic activity has been found (see below, Functional Domains).

Secondary Structures

Analysis of the secondary structural elements (α-helix, β-sheet, β-turn, and random coil) of the NT types A, B, and E revealed that these proteins have highly ordered structures that are dominated by the β-sheets. About 62–72% of the amino acid residues are in the ordered structure at pH 7.2 (21–28% in α-helices plus 41–44% in β sheets), and the rest are in random coils (39). At pH 5.5, which approximates the pH inside the endosomes, the type A NT also retains the highly ordered structure: α-helix 29%, β-sheet 45–49%, and random coils 22–26% (40). The 29% α-helix content of the NT at pH 5.5 compared with 21% at pH 7.2 may reflect the acid-induced conformational change the NT presumably undergoes inside the endosome before channel formation (see below). Analysis of the L and H chains of type A NT following their chromatographic separation gave an interesting insight into their conformational stability (41). The sums of the α-helix, β-sheet, β-turn, and random coil contents of the separated L and H chain, as a weighted mean, were similar to the content of the corresponding structural elements in the NT (see Table 1), e.g., the sum of the α-helix content of the L chain (22%) and the H chain (18.7%), 19.8% as a weighted mean, was similar to the α-helix content of the NT (20%). In other words, the secondary structures of the L and H chains do not change significantly when they are separated. This stability indicates that the two chains, by virtue of their structural integrity, may express their individual biological activities even when physically separated. This notion agrees with two independent experimental observations: (1) The two chains can be separated and then reconjugated to form disulfide-linked NT (mol. wt. 150,000), which is highly active (see references, in Ref. 28). (2) The separated chains, although nonlethal by themselves, are biologically active, i.e., the isolated H chain forms channels in lipid bilayer membranes and binds to the receptor; the isolated L chain presented to the interior of a neuronal or chromaffin cell inhibits secretion of neurotransmitter (see references in Ref. 42).

Table 1 Secondary Structure Elements of Type A Neurotoxin and Its Light (L) and Heavy (H) Chain After Separation

Protein	α-Helix	β-Sheet	β-Turn	Random coil
Neurotoxin (mol. wt. ~150,000)	20.0	37.5	15.2	27.2
L chain (mol. wt. ~50,000)	22.0	27.5	18.7	31.7
H chain (mol. wt. ~100,000)	18.7	40.0	13.0	28.2
Sum of L and H chain (weighted mean)[a]	19.8	35.8	14.9	29.4

These secondary structure elements were obtained from circular dichroism spectra (240–200 nm) of the protein (0.1–0.3 mg/ml) in 10 mM sodium phosphate buffer, pH 8.1, containing 100 mM NaCl, at 23–25°C.
[a]Calculated as (1 × L chain + 2 × H chain)/3 because the H chain is twice the size of the L chain.

Tertiary and Quaternary Structures

A simple model of the folded configuration of the NT has been built based on the results of limited proteolysis (33). Figure 3, which incorporates the ideas presented in Fig. 1b, shows the common narrow regions of the NT serotypes A, B, and E cleaved by various proteases (33). Three of these regions, sites 1, 3, and 4, are highly susceptible. Site 1, the nicking region, is about one-third of the distance from the N-terminus; site 3 is approximately at the middle of the H chain, and site 4 is near the C-terminus. Figure 1b has depicted (1) that following cleavage at site 1, the L chain remains bound to the N-terminal half of the H chain by disulfide and noncovalent bonds (dotted lines between the L and H chains); (2) that noncovalent interactions between the L chain and the C-terminal half of the H chain are virtually absent or extremely weak; and (3) that association between the two halves of the H chain is also absent or very weak. Thus cleavage at site 3 allows the C-terminal half (mol. wt. ~ 50,000) to separate from the rest of the molecule (mol. wt. ~ 100,000) very easily (see references in Ref. 33). The C-terminal half of the H chain (result of cleavage at site 3) has been found completely or extensively digestible by trypsin, chymotrypsin, and pepsin. The N-terminal half of the H chain, on the other hand, survives proteolysis remarkably (reviewed in Ref. 33). Proteolytic digestions of NTs carried out in various laboratories have not reported significant cleavage of the L chain. This suggests (1) that the L chain and the N-terminal half of the H chain probably are individually resistant to proteolysis and/or (2) that the association between these two segments of the NT makes their protease-susceptible sites unavailable for cleavage.

Many observations indicate that the natural foldings of the polypeptides protect the proteins from proteolytic assaults, that limited proteolysis of native proteins is usually restricted to interdomain regions, and that these susceptible regions are flexible hinges on the protein surface. Consistent with this view are the proteolysis-susceptible regions on the NT detected so far. The two halves of the H chain (generated by cleavage at site 3), the N-terminal and C-terminal halves, also retain after separation their channel-forming (43,44) and ganglioside-binding activities (45), respectively. Earlier it was noted that the L chain separated from the H chain retains biological activity. Thus, the three segments of the NT that exhibit biological function after separation, the L chain and the two halves of the H chain, each of ~50,000 mol. wt., represent three domains spaced in the polypeptide backbone by two hinges (indicated in Fig. 3b) and proteolytically susceptible regions. (These segments were referred to as A-C-B above, "Introduction".)

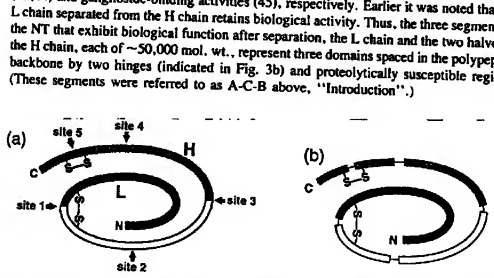

Figure 3 Model of folded configuration of the neurotoxin (NT) based on proteolytic digestions. (a) Sites on NT types A, B, and E cleaved by various proteases (reviewed in Ref. 33). (b) Flexible hinges (the proteolytically susceptible regions) of the NTs, spaced in the polypeptide backbone, are indicated as wasp-waist segments.

The above observations allowed us to build the model in Fig. 3 (33) the salient features of which are as follows: (1) sites 1, 2, 3, and 4 are on the surface and hence accessible to proteolysis, (2) most of the L chain is shielded by the H chain from proteolysis, (3) most of the N-terminal half of the H chain is closely associated with the L chain, (4) the C-terminal half of the H chain is not associated with the rest of the molecule, and (5) the C-terminal segment is highly accessible to receptors on the target cells (see below) and proteolysis, and it allows the NT molecules to associate to form what appear to be "dimers" (see below). The shape of the NT molecule is ellipsoid rather than spherical, so that one of its axes is longer than the others (the reasons are discussed in Ref. 33). The diameters of the NT serotypes A, B, and E, measured by dynamical light scattering, are 100 ± 4, 110 ± 4, and 100 ± 4 Å (46). The diameter of the type A NT, 96 Å (stokes radius = 48 Å), measured many years ago by gel filtration (17), agrees well with the new data. It is not clear yet if this diameter represents the size of the NT monomer (mol. wt. 150,000) or dimer (mol. wt. 2 × 150,000).

Three independent lines of evidence indicate that the NT molecules associate to form entities larger than 150,000 (mol. wt. 300,000 and larger): (1) Chromatography of pure type A NT yielded type A NT molecules larger than 150,000 (47). (2) Polyacrylamide gel electrophoresis (without SDS) of pure type A NT demonstrated protein species of mol. wt. 300,000 and larger (33,43,48). The NT without the C-terminal half of the H chain (i.e., after cleavage at site 3) does not associate to form larger-molecular-weight species. (3) Crystals of pure type A NT also indicate dimerization (49).

The pure type of A NT (mol. wt. 150,000) has been crystallized in three different crystal morphologies; all three have the same crystal form and diffract to 3 Å (49). Determination of the three-dimensional structure of botulinum NT at atomic resolution now appears an achievable goal.

Functional Domains

The absolute neurospecificity and extremely high potency of the NT are attributable to its high affinity for specific receptors on the presynaptic membranes and to an enzymatic action, functions of the H and L chains of the NT, respectively (Fig. 4). To explain how the NT at extremely low concentrations can bind specifically to the nerve cells, the proposal of Montecucco (50) deserves reiteration. The NT first binds to the ganglioside-rich lipid membrane, then the lipid-NT complex moves laterally to reach and bind the NT-specific receptor, which is protease-sensitive. Accordingly, any docking of the NT molecule on the membrane results (following the "catch-and-delivery effect") in a productive binding with the NT-specific receptor protein. The lipid-binding step "is actually equivalent to concentrating the NT and its protein receptor in a much smaller volume . . . because the partners of the binding reaction are now restricted to the two-dimensional plane of the plasma membrane rather than in the three-dimensional water phase" (50). The two-step hypothesis agrees with experimental results. Two-dimensional, ordered arrays of NT types A and B form at the interface of a NT solution and phospholipid monolayer containing the ganglioside GT_{1b}. The NT binds the hydrophilic moiety of the ganglioside, and two-dimensional diffusion allows crystals to form (51).

Receptors (also referred to as acceptors) of high affinity have been identified (the K_D values, for example, for type A, B, and E NT range from 0.5 nM to 100 pM; see Refs. 2,15,42 for review). In fact, two receptor species, one with higher affinity and low populations, and another with lower affinity and higher populations, have been identified for type B NT (52). Some NT serotypes do and some do not share the same receptors;

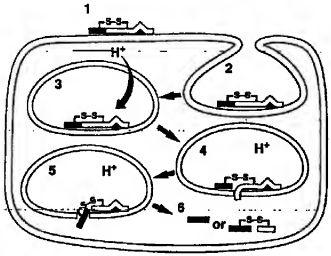

Figure 4 A simple diagrammatic representation of the sequential events that lead to the blockade of neurotransmitter release by the neurotoxin (NT). The NT binds to the receptor (black triangle) on the presynaptic membrane (step 1) via its heavy (H) chain. Endocytosis (step 2) internalizes the NT, which is now inside the endosomes (step 3). The acidic pH inside the endosome (steps 3,4) induces formation of channels in the endosomal membrane by a segment of the H chain (step 4) that allows the light (L) chain to egress from the vesicle (step 5) into the cytosol (step 6), where the L chain acts enzymatically and disrupts the neurotransmitter secretion. This model accommodates the possibility that the single-chain NT, after binding the receptor and internalization, is nicked to the dichain by the endosomal proteases (between steps 3 and 4). The model does not attempt to define the step at which the disulfide bond between the L and H chain is broken.

e.g., types A and E share the same receptor, types A and B do not (53). The densities of the distinct populations of receptor/acceptor (mouse diaphragm) for NT types A and B have been determined; their average numbers are 153 ± 21 and 637 ± 131 per μm^2 of membrane, respectively (54). Black and Dolly (54) considered these densities too high relative to the number of NT molecules needed to induce in vivo paralysis (1 mouse LD_{50} being equivalent to $\sim 1.2 \times 10^{-11}$ g or 8×10^{-17} mol or 5×10^{7} molecules of type A NT; at the maximum, 100–1000 molecules are needed to block neurotransmission at each synapse). Thus the receptor/acceptor densities greatly exceed the number of NT molecules needed to block a synaptic transmission, arguing against a "one-hit" model for the NT's mechanism of action. The role of high densities of acceptors therefore appears to be to concentrate the NT on the target membrane surface for the next step—delivery inside the cell via intracellular vesicular compartments.

The ganglioside (GT_{1b})-binding site on type A NT is primarily confined within the C-terminal (50,000 mol. wt.) half of the H chain; the N-terminal half of the H chain did not bind GT_{1b} under any conditions tested, and the L chain exhibited a small degree of binding, which might be nonspecific (45). The demonstrated ganglioside-binding site on type A perhaps can be generalized for the other serotypes. Analogy with tetanus NT, which is very similar to botulinum NT in structure, structure-function relationship, and mechanism of action (2,15,55), further indicates the receptor-binding role of the C-terminal half of the H chain. A protein (mol. wt. ~20,000) expressed by PC12 cells following differentiation with nerve growth factor binds tetanus NT, and the binding is neuro-

specific. Only the C-terminal half of the H chain of tetanus NT binds to this protein; the remaining segment of the NT, i.e., the L chain and the N-terminal half of the H chain (mol. wt. ~100,000), did not bind (56 & reference 33 therein). These experiments once again demonstrate the role of the C-terminal half of the H chain in receptor binding. A protein(s) that appears to act as the receptor protein for botulinum NT type B has been reported (52,54).

According to the currently held view, when the pH inside the endosomes drops, the NT entrapped within this acidic environment undergoes some conformational change leading to an insertion of a segment of the NT into and across the endosomal membrane (Fig. 4, step 4). This poorly understood process somehow allows the L chain or the L chain and a segment of the H chain to cross the membrane and reach the cytosol. The experimental evidence behind this scenario is low pH–induced channels in lipid bilayers formed by the H chain and the N-terminal half of the H chain. Neurotoxin added to one side of an artificial lipid membrane forms few channels when on both sides the pH is ~7.0 or ~5.0; however, when the pH on the side of the NT is lowered to ~5.0 and kept near 7.0 on the other side, many channels are formed. The channel-forming activity is confined to the N-terminal half (mol. wt. ~50,000) of the H chain (43,44, and references therein). This pH gradient favorable to channel formation mimics the condition of low pH inside the endosome and physiological pH of the cytosol, i.e., outside the endosome. A narrow segment of the N-terminal half of the H chain of type A NT, residues 650–681, has been located that appears to be responsible for channel formation (57). Whether these channels provide a large enough opening for a polypeptide to pass through is not yet known.

Enzymatic activity of NT was recently demonstrated based on the proteolytic cleavage of a neuronal protein (58). Incubation of highly purified small synaptic vesicles (rat cerebral cortex) with the NT serotype B cleaved a single peptide bond (between residues 76 and 77, Gln-Phe) of synaptobrevin-2 (also called VAMP), which is a synaptic vesicle–associated integral membrane protein. Of the two isomers of synaptobrevin, synaptobrevin-1 has Val in the position of Gln; the Val-Phe bond in synaptobrevin-1 was not cleaved. The L chain of tetanus NT also cleaved synaptobrevin-2, and thus by analogy the proteolytic activity of botulinum NT is confined in its L chain. Unlike the type B NT, type A and type E NTs did not show any cleavage (58).

The nonidentical actions of NT types A, B, and E are not surprising; the intracellular inhibitory effects of the type A, B, and E NTs studied in permeabilized chromaffin and PC12 cells also show notable differences. The Ca^{2+}-stimulated secretion of norepinephrine was inhibited most by type E and least by type A (26,27). Long before permeabilized secretory cells were utilized to study the intracellular inhibitory actions of NT, Sellin (53) had proposed, on the basis of other experimental evidence, that various NT serotypes do not follow a single mechanism of action, and that the intracellular site of action of type A is distinct from those of types B, E, and F. (See Note Added in Proof.)

Potential Use of Different Neurotoxin Serotypes and Chimeric Neurotoxins

The different NT serotypes could be used clinically to exploit their nonidentical pharmacological actions rather than only to obviate the immunity that may develop from repeated administration of a single serotype. This idea must have crossed many minds. Further additions to the repertoire of pharmacological differences may be made by generating chimeric neurotoxic molecules, e.g., an NT made of L chain of type E and H chain of type A. The following considerations of the structures and structure-function relationships of the NTs favor the above two ideas. The different paralytic effects (magnitude, rapidity, duration, and recovery/reversal) produced in identical neuromuscu-

lar preparations by different NT serotypes (e.g., type A vs. type E, in Refs. 59,60) are probably rooted in the intrinsic structural features of the functional domains of the NT (42) and some of the components of the target neuronal cells. The population of receptors specific for the different NT serotypes present on the neuromuscular junctions of various muscles may not be identical. A therapeutic target area X may be significantly richer in receptors for NT type A than for types B and E, and other target areas Y and Z may have more receptors for NT type B and E, respectively. Experimental knowledge of such differences would indicate that better tools to paralyze muscles at target areas X, Y, and Z could be NT types A, B, and E, respectively. This consideration, based on the function of the H chain, attempts more efficient capturing of the NT and delivery of it inside the target neuronal cells. A corollary of this approach is that a lower amount of administered NT protein also lowers the immunogen load. The actual inhibition of neurotransmitter release could be further manipulated by presenting the target cells' cytosol with an L chain, chosen on the basis of its intracellular inhibitory activity. Thus a chimeric NT can be designed and made from L and H chains from two different NT serotypes, each chosen on the basis of its functional properties. Certain combinations of these two structures could provide therapeutic agents more suitable than the NTs we know of now.

Production of chimeric NT is clearly feasible. The L and H chains of NTs after separation can be reconjugated to generate neurotoxic dichain NT (mol. wt. ~150,000, see references 4–6 in Ref. 61). The chemistry involved in this approach has allowed generation of type A NT that was selectively radiolabeled at either the L or the H chain (one of the separated chains was radiolabeled and then combined with the corresponding nonlabeled chain) (61). More convincing is that the L and H chains of tetanus NT have been combined with H and L chains of botulinum type A NT, and that the chimeric NTs (part tetanus, part botulinum) exhibited predicted biological activities (62,63).

CRYSTALLINE TYPE A TOXIN

The preparation of type A NT that has found rapid and wide acceptance for therapeutic use is the crystallized mixture of type A NT and nonneurotoxic proteins; since 1967 (64) this complex material has hardly received a rigorous analytical scrutiny satisfactory to the standards of modern protein biochemistry. Crystallographic data for the preparation have never been reported.

The following properties of the crystallized toxic preparation are used to judge its purity (19):

1. Around neutral pH it absorbs maximally at 278 nm.
2. The ratio of its absorbance at 260 and 278 nm is 0.6 to 0.55.
3. An absorbance of 1.65 at A_{278} is equivalent to 1 mg protein/ml.
4. Its isoelectric point is 5.6, and at acidic pH it migrates in electric field as a single band.
5. The nitrogen content is 16.2%.*
6. It contains about 0.1% or less RNA.
7. Its specific toxicity is $3 \times 10^7 \pm 20\%$ LD_{50}/mg protein.

The properties listed as items 1, 2, 3, and 5 are not unique features of this protein preparation because these could be parts of general properties of proteins.

* This is old data (65), although "the nitrogen content of the toxin was redetermined and found to be 16.08%" (64).

Comments on the Optical Properties

The three aromatic amino acids tryptophan, tyrosine, and phenylalanine, in aqueous solution, absorb light at wavelengths of 250–300 nm in characteristic fashions. Tryptophan, tyrosine, and phenalalanine maximally absorb near 280, 275, and 260 nm, respectively. Thus the absorption profile of a protein in aqueous solution, in the region 250–300 nm, is determined both qualitatively and quantitatively by the absolute number of the three aromatic amino acids and their relative proportion. A protein completely free of nucleic acid (which absorbs maximally near 260 nm) can have significant absorption at 280 and 260 nm, the relative extent of which depends on the characteristic amino acid composition.

The absorption profile of pure type A NT (Fig. 5) shows an absorption maximum at 278.2 nm, a minimum at 249.5 nm, and a steep rise below 250 nm. (Proteins, like many organic compounds, also extensively absorb below 250 nm.) The pure type B and E NTs, prepared in our laboratory, exhibited 278.0 and 277.7 nm as absorption maxima and 249.7 and 250.0 nm as absorption minima, respectively (Table 2). The ratios of the absorption maxima and minima for these type A, B, and E NTs were 0.301, 0.301, and

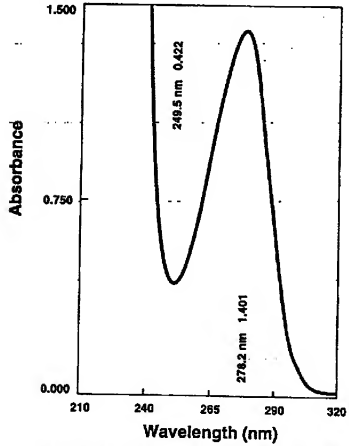

Figure 5 Absorption profile of pure type A neurotoxin (mol. wt. 150,000) in 0.1 M sodium phosphate buffer, pH 7.4, plus 0.05 M NaCl (UVIKON 860 Spectrophotometer, Kontron Instruments).

0.286, respectively. The ratios at A_{260}/A_{278} of the NTs purified by chromatography are invariably below 0.5.

The theoretical molar extinction coefficient of type A NT (mol. wt. 150,000) at 280 nm, calculated from the molar extinction coefficients of tryptophan, tyrosine, and phenylalanine and their known abundance in the protein (15, 74, and 70 residues, respectively, see ref. 3 in 31) is 181,150, which means that 1 mg/ml of the NT has an absorbance of 1.212 at 280 nm (B. R. DasGupta, unpublished data). The large difference between the deduced absorbance of the pure NT, 1.21, and that of the crystalline complex, 1.65, is probably due to the nonneurotoxic components in the complex.

The notion that "the purity of the crystalline toxin cannot be defined strictly in terms of percent purity because of small amounts of undefined material absorbant at 260 nm, most probably nucleic acid material" (19) is not completely right, because as mentioned above any protein with aromatic amino acid residue absorbs at 260 nm even if nucleic acid is entirely absent. Thus the use of the ratio of absorption at 260 and 278 nm has very limited value in judging whether a protein is free from other proteins and nucleic acid. This point is also articulated by the data in the last column in Table 2. Note that any mixture of pure type A, B, and E NT, in any proportion, will have a ratio of absorbance of ~0.50 at 260 and 278 nm.

Electrophoretic Analysis and Criteria for Testing Purity

The electrophoretic migration of the crystallized type A toxin preparation as a single band at acidic pH, an observed fact, has questionable value for judging the purity of the protein preparation. Although it has been known since 1980 (66) that the nonneurotoxic components that form a complex with type A NT can be resolved electrophoretically in the presence of SDS into at least 7 proteins, some of which were partially sequenced recently (67), this author is not aware that the crystalline preparation has been defined as to the exact number of proteins it is composed of and their relative proportion. The basis of the idea that the crystalline toxin (mol. wt. 900,000) "is composed of two molecules of neurotoxin (ca. 150,000 M_r) non-covalently bound to non-toxic proteins" (19) was not described and is not known.

Consideration of the purity of a substance requires a definition of the substance; the more exact the definition of its composition, the more meaningful consideration of its purity becomes. Many biologically active proteins composed of multi-subunits that are homomers and/or heteromers (a few examples are ribosome, ATPase, acetylcholine receptor, hemoglobin, pertussis toxin, and cholera toxin) have been precisely defined.

Table 2 The Ultraviolet Absorption Properties of Botulinum Type A, B and E Neurotoxins

Neurotoxin	Absorption		Ratio of absorption	
	Minimum (a)	Maximum (b)	at a/b	at 260/278 nm
Type A	249.5 nm	278.2 nm	0.301	0.491
Type B	249.7	278.0	0.301	0.495
Type E	250.0	277.7	0.286	0.468

Type A NT in 0.1 M Na-phosphate buffer pH 7.4, plus 0.05 M NaCl.
Type B NT in 0.1 M Na-phosphate buffer pH 8.0, plus 0.05 M NaCl.
Type E NT in 0.1 M Na-phosphate buffer pH 7.4, plus 0.01 M NaCl.

The published literature does not inform us exactly how many different proteins (and nucleic acids) combine in what relative proportion with the NT to form the complex that is eventually crystallized. This understanding needs to be developed rigorously before the issue of purity of the crystallizable complex can become meaningful. If, for example, it can be shown that the crystallized complex is made of NT and, let us say, seven different nonneurotoxic proteins, and all eight proteins combine in certain fixed relative proportion (e.g., 1:1:1:1:1:1:1:1), then the purity of the crystallized complex can be qualified according to the presence of anything in addition to the NT and the hypothetical seven other proteins.

Simple experimental techniques are available to develop this information objectively. Electrophoresis of the complexes of NT and nonneurotoxic proteins in polyacrylamide gel in the presence of SDS resolves the complexes into multiple bands that can be visualized after staining with Coomassie blue or silver (see Ref. 67 for such patterns from type A, B, and E complexes). The total number of different proteins present in a complex and their molecular weights can thus be delineated. Densitometric scanning of the band patterns in such gels provides a dependable quantitative profile of the protein components and thus of their relative proportion in the complex.

Toxin Complex and Crystalline Toxin A, History and Current Status

In the bacterial culture, the NT exists as a large complex made of the NT (mol. wt. ~150,000) and nonneurotoxic protein(s); noncovalent association keeps the proteins together (Fig. 6). The nonneurotoxic protein(s), which seems to be produced by the bacteria simultaneously with the NT, may or may not agglutinate red blood cells, i.e., may or may not have hemagglutinating (Hn) activity. Based on this property these nonneurotoxic proteins have been designated Hn^+ and Hn^- (67).

Attempts made in the mid-1940s to purify the NT from the bacterial culture resulted in the isolation of a complex of the NT and nonneurotoxic proteins, which were crystallized in 1946 by two groups: Lamanna, McElroy, and Eklund (9) and Abrams, Kegeles, and Hottle (11). The molecular weight of this complex is 900,000. Duff et al. (68) modified the earlier protocols for partial purification of the NT (9,10,11) and also obtained a complex that crystallized. This modified protocol, developed in 1957 (68), was used to prepare the crystallized mixture of type A NT and nonneurotoxic proteins that was introduced for therapeutic use (18,19). The mixture of NT, other proteins, and nucleic acid that crystallizes readily was obtained entirely by differential precipitation steps (9,10,14,68) and without the benefit of high resolution achieved by chromatography. Anion-exchange chromatography of the crystalline type A toxin separated the NT from the nonneurotoxic proteins; only about 20% of the protein in the crystalline preparation was found in the NT (16,17).

Thus the preparation may well be called crystalline hemagglutinin rather than crystalline toxin. The alternative name, perhaps frivolous, is more apt simply because the weight of the argument is against the NT content in the preparation. Referring to the crystalline type A botulinum toxin, Lowenthal and Lamanna (69) wrote that "work on the characterization of the type A botulinal toxin was in reality characterization of the toxin-hemagglutinin complex, rather than of toxin alone." The preparation of type C toxin complex that was crystallized (70,71) contains hemagglutination activity, and polyacrylamide gel electrophoresis (without SDS) revealed that it is a mixture of at least three proteins (70).

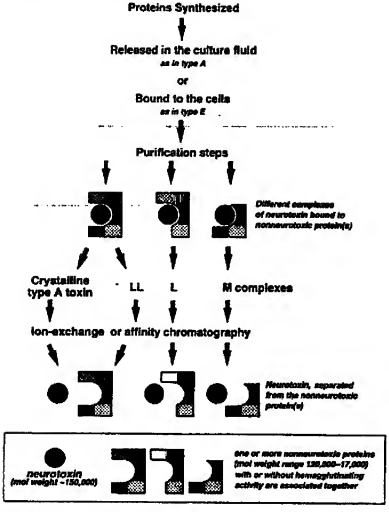

Figure 6 Schematic description of formation of complexes between the neurotoxin (mol. wt. ~150,000) and nonneurotoxic proteins and their eventual separation by chromatography.

The protocol in use since 1957 for isolating type A toxin complex as crystals (68) was modified in 1977 (72). In this improved protocol, one chromatography step was introduced that substituted precipitation of the toxin complex by ethyl alcohol. There were three beneficial results: (1) a twofold greater recovery (33% vs. 17%) of the total toxicity present in the bacterial culture was gained; (2) more effective removals of pigments and nucleic acids (the A_{260}/A_{278} ratio was 0.52) were achieved; and (3) the crystal lots produced were of greater uniformity.

Crystals of the mixture of proteins produced according to the improved procedure (72) and by following Duff et al. (68) contained three antigens (detected by double-diffusion serology with anti–crystalline serum), one of which was the NT (detected by anti–pure NT serum). The minor antigen was eliminated from the crystals produced by either method following recrystallization (72).

Sugii and Sakaguchi (73) had concluded earlier that "the generally accepted notion that type A crystalline toxin consists of two components, neurotoxin and hemagglutinin,

will have to be changed." They isolated, using chromatography, two kinds of complexes, called L and M (with specific toxicity of 2.5–3.0 × 10^8 and 4.5–5.0 × 10^8 mouse MLD/mg N, respectively). Both were composed of at least three distinct components, NT, hemagglutinin, and an inert protein. The nonneurotoxic component of M complex contained little or no hemagglutination activity. The nonneurotoxic materials in crystallized toxin and L complex consisted of two distinct antigens and were antigenically identical. The inference was that L complex and crystallized toxin are similar in composition.

The two reports (72,73) indicate that methods using chromatography are available to isolate complexes of NT and nonneurotoxic proteins that in purity are at least equivalent to or better than the crystallizable product produced following the protocol of Duff et al. (68).

Type B NT was also initially isolated as a complex of NT and nonneurotoxic proteins (74); the molecular weight of the large complex was found to be 500,000 (75). Modification of this purification protocol that did not include any chromatographic step yielded a preparation that appeared as a single entity and a large complex (the S_{20w} value was 12.7) by ultracentrifuge analysis (76). Only ion-exchange chromatography could resolve the complex into the NT (initially reported as 165,000 mol. wt.) and the nonneurotoxic proteins (77,78).

Neurotoxin serotypes A and B do not serologically cross-react. Some of the nonneurotoxic proteins associated with type A and type B NTs do cross-react. This has produced a great deal of false data and confusion. Antiserum produced against the complex of type A does not neutralize type B NT (and vice versa) when the assay is mouse lethality. However, the same polyclonal antiserum (i.e., produced against the complex of type A) shows positive reactions in Ouchterlony, ELISA, and RIA assays against a type B NT preparation if it has a detectable amount of the nonneurotoxic Hn^+, Hn^- proteins. These problems can be avoided by producing the polyclonal antiserum against the pure NT completely free of the associated nonneurotoxic proteins. Cocrystallization of two or more substances (organic or inorganic) is a common phenomenon, and it is not peculiar to proteins. Repeated recrystallization of such a mixture using the same or different solvents often generates purer crystallized material, generally at the cost of yield, but also often produces a mixture whose composition remains constant, and thus further selective enrichment of one component cannot be attained by the same approach (azeotropic mixtures and eutectic compositions present analogous situations).

Attempts to improve the quality of the material in the crystallized complex of type A NT by recrystallization appear to have reached the point of diminishing returns. This was recognized as early as 1946, when Lamanna and colleagues (10) wrote: "Amorphous, crystalline, and recrystallized materials have been determined to have the same toxicity within the limits of errors of mouse titration." When the ratio of absorption at 260 and 278 nm was used as a parameter of purity (and as an indicator of the presence of nucleic acid) it was close to 0.6 and near 0.55 at the end of the first and second recrystallization steps, respectively. The third crystallization reduced the ratio only slightly, with a considerable loss of the material (19). The purity of the crystallizable proteins is likely to improve if separation techniques different from the ones currently used (19,68) are employed to break out of the "constant composition deadlock." Introduction of a simple chromatographic step, as mentioned above (72), in the old protocol (68) of preparation of the type A NT complex has indeed reduced the A_{260}/A_{278} ratio to 0.52 and increased the yield of the final product by a factor of 2.

Detoxification of Crystalline Type A Toxin and pH

A recent carefully conducted study (79) found that the freeze-dried crystalline type A toxin (used clinically), which is stable for months under refrigeration, was 43.9% detoxified (statistically significant) after reconstitution for clinical use (50 ng toxin, 500 μg human serum albumin, and 900 μg NaCl at pH 7.3) and storage at 6°C for 12 hr but was not detoxified for the first 6 hr. The authors of this study, noting that the toxin preparation is more stable in solution at pH 6.2 than at pH 7.3, suggested, justifiably, that the reconstituted toxin be maintained at pH 6.2 rather than at pH 7.3. The time-dependent inactivation of the toxin preparations above pH 7.0 was observed by the early workers* and probably for this reason, in many of the past studies with crystalline type A toxin the pH was maintained strictly below 7.0.

One likely reason for the pH- and time-dependent inactivation of the toxin preparations is the possible presence of traces of protease(s) whose activity is suppressed at acidic pH and is high near pH 7.3. This reasoning is favored by the following considerations: (1) The pH- and time-dependent inactivations of toxicity were observed primarily when the neurotoxic preparations were not chromatographically purified. (2) After chromatographic purification, the type A complex could be exposed to pH above 7.0 without notable loss of toxicity (73,80). (3) The pure NT is stable for months at pH 7.9 and 4°C (81). The postulated pH-dependent inactivating agent, probably a protease, therefore appears to be separable from the NT if chromatographic steps are employed.

The rationale for clinical use of the impure type A NT (about 80% of protein in the crystallized complex is nonneurotoxic protein) is that the nonneurotoxic proteins "bound to the neurotoxin apparently play an important role in maintaining the toxic shape of the neurotoxin" (19). If this is so, why then does the diluted crystallized preparation need to be fortified by adding gelatin or serum albumin to maintain neurotoxicity, as is commonly done (18,19)? Does this suggest that the pure NT (mol. wt. ~150,000), separated from the nonneurotoxic proteins, may also be fortified from detoxification with some other clinically acceptable stablizing agent(s)? Such a preparation concocted with a known concentration of the pure NT and a defined added entity (for storage) could have a precisely determined composition.

Progress in this direction is not evident from publications, perhaps because of the assertion that "an important point regarding the use of purified neurotoxin besides its instability is the fact that it cannot be prepared with constant composition and activity" (18). This is not true, and this prevailing view needs rectification. The NT (mol. wt. ~150,000) isolated from the complex by ion-exchange chromatography, first reported in 1966 (16) and now routinely prepared in various laboratories (73,48), has stable activity;

*(a) "The toxin . . . is extremely sensitive to pH values above 6.5 to 7.0 at room temperature" (10). (b) ". . . above pH 7.0 the toxin was rapidly destroyed" (11). (c) "Rapid inactivation takes place in solutions above pH 7.0" (82). (d) During purification of the toxin the pH was not allowed to go above 6.5 (9). (e) The highest pH attained during purification of the toxin by Duff et al. (68) was 6.8. (f) Chromatography of the crystalline toxin on DEAE-cellulose was attempted at pH 6.5, and its fluorescence was studied at pH 6.8 (83). (g) Standardization of the crystalline toxin's mouse lethality was done in solutions of pH 6.2 (84). (h) The toxicity of a batch of crystallized type A toxin was found stable at pH 8.0 and 9.0 at 5°C for over 2 months (85). The method used to isolate the toxin appears different from that of Duff et al. (68), but the exact details were not given (85).

Williams et al. (81) have found that the homogeneous preparation of type A NT, stored at 4°C in 0.15 M TRIS-HCl buffer, pH 7.9, is stable for several months. The pure preparations of NT types A and E at very low concentrations (such as 1×10^{-10} M and lower) in physiological buffers, with and without added gelatin or serum albumin, are highly active (25,48,59,73,86). The effect of long-term storage on the pure NTs at high dilutions is not apparent in the published literature. The NT has constant physicochemical composition. Amino acid analysis of multiple batches of type A NT gave reproducible composition (87), and the same is true for type B and type E NTs (see refs. in 88). The amino acid compositions of the L and H chains after separation also show constancy; the sum of the amino acid contents of the two subunits equals the amino acid content of the NT (88). Table 3 shows (1) that the total number of the amino acid residues of the L and H chain of type A, within experimental error, is equal to that of the NT, and (2) that the amino acid composition determined empirically from acid hydrolysis of the protein matches, within experimental error, the composition derived from the amino acid sequence of the NT predicted from nucleotide sequence. Furthermore, those segments of the

Table 3 Number of Amino Acid Residues in Type A Neurotoxin (NT) and the Separated Light (L) and Heavy (H) Chains Determined by Amino Acid Analysis, and Comparison with the Amino Acid Residues of the NT Predicted from Nucleic Acid Sequence

	From amino acid analysis (Ref. 88)				From sequence (Ref. 31)
	L	H	H+L	NT	NT[a]
Asp	65	149	214[c]	200	78
Asn[b]	—	—	—	—	137
Glu	39	79	118	114	76
Gln[b]	—	—	—	—	39
Ser	28	55	83	79	84
Gly	30	37	67	64	64
His	6	6	12[d]	14	13
Arg	15	30	45	43	43
Thr	33	39	72	75	71
Ala	20	35	55	53	54
Pro	20	21	41[c]	44	38
Tyr	25	49	74	71	74
Val	26	40	66[c]	70	72
Met	7	17	24[c]	22	23
Ile	30	82	112	111	119
Leu	40	76	116[d]	104	113
Phe	34	37	71	68	70
Lys	39	63	102	100	103
Cys	4	6	10	10	9
Trp	4	16	20[d]	17	15

[a]The NT sequence, predicted from the nucleotide sequence, gives the amino acid composition of the single-chain protein before nicking. After nicking, 10 residues are excised (Krieglstein, DasGupta, and Henschen, to be published); subtraction of Thr-Lys-Ser-Leu-Asp-Lys-Gly-Tyr-Asn-Lys from this column makes agreement between this and the preceding column closer.

[b]Asn and Gln were determined as Asp and Glu after acid hydrolysis.

[c,d]Deviations between the sum of amino acid residues of H and L, and the parent NT: >5 and <10%,[c] >10%,[d] all others <5%.

NT that have been analyzed for amino acid sequence (based on direct protein sequencing) match with the sequence predicted from the nucleotide sequence (not shown in the table). Also, the sum of the contents of secondary structure elements (α-helix, β-sheet, etc.) of the separated L and H chains equals the content of such elements of the parent molecule; thus the structural domains of the L and H chains are stable.

Toxicity Assays

In a very well conceived plan, the mouse lethality assay of the crystallized type A toxin was rigorously evaluated (84). Using one standardized toxin preparation, assays carried out in 11 independent laboratories according to a single prescribed protocol gave an average value of 0.043 ng toxin equivalent to 1 mouse LD_{50} (the highest and lowest values were 0.075 and 0.032 ng, respectively; standard deviation was 0.012). Thus, 1 ng toxin is equivalent to 23.2 LD_{50}. This has been regarded as the recommended standardized potency of the toxin preparation and assay procedure (19), yet this same publication (19) defined 1 ng as equaling 30 mouse LD_{50} without accounting for the difference between the values of 23.2 and 30 LD_{50} per ng toxin.

The range in mouse lethality results noted in the 11-laboratory study was thought to be due to variation in the response of mice to toxin in each laboratory (84). In this context, consideration of the importance of correct placement of the inoculum during intraperitoneal injection appears relevant. Studies with substances other than botulinum toxin have revealed a 14% (89), 10–20% (90), and 12% (91) error in the placement of intraperitoneal injection of mice with a one-person procedure of injection. All or part of the misplaced inoculum was injected into the lumen of the stomach, the small bowel, or the uterine horn, or was injected subcutaneously, retroperitoneally, or intravascularly. The incidence of error was consistently reduced to 1.2% with a two-person procedure of injection (91).

The assertion that "the only means of evaluating the potency of acetylcholine-blocking power of the toxin is an animal assay [and that] there is no known . . . biological or immunological test available that can replace the mouse test for toxicity evaluation" (19) seems to preclude exploitation of an important alternative approach that actually monitors muscle paralysis, i.e., assessing the immediate postsynaptic effect of poisoning within 3 hr rather than recording the number of mice dying up to 72 hr.

Nerve-muscle preparations used for electrophysiological studies of neuromuscular junctions are useful for assaying the paralyzing effects of botulinum NT. Besides the classic phrenic nerve–hemidiaphragm (14), the plantar nerves–lumbrical muscles of the hind paw of the mouse (86) and the chick ciliary ganglion–iris muscle preparation (59) have been tested for their response to type A and E NTs. Some of these data, including the comparative response of phrenic nerve–hemidiaphragm preparations from various animal species to type E NT, are summarized in Table 4. In these nerve-muscle preparations the relationship between paralysis time (the time elapsed from addition of NT to tissue bath to loss of neurogenic response) and NT concentration (within a certain range) was linear or approximately linear. For example, when the crystalline type A toxin and rat phrenic nerve–hemidiaphragm were used, a plot on logarithmic coordinates of toxin concentration (3×10^{-9} to 1×10^{-11} M) versus paralysis time (80–300 min) yielded a straight line (14). Paralysis time (36–145 min) of mouse plantar nerve–lumbrical muscle was approximately linearly dependent on the concentration range of pure type A NT (1×10^{-9} to 1×10^{-11} M). The chick ciliary ganglion–iris preparation also exhibited muscle paralysis

Table 4 Paralysis of Nerve-Muscle Preparations by Various Concentrations of Type A and E Neurotoxins

Toxin	Toxin Concentration (M)	Paralysis time (min)	Species and tissue	Reference
Crystalline type A toxin	3×10^{-9}–1×10^{-11}	80–300[a]	Rat phrenic nerve–hemidiaphragm	(14)
Pure type A neurotoxin	1×10^{-9}–1×10^{-11}	36–145[b]	Mouse plantar nerve–lumbrical muscle	(85)
Pure type A neurotoxin	1×10^{-7}–1×10^{-9}	24–80[c]	Chick-iris	(59)
Pure type E neurotoxin[d]	3.4×10^{-10}–3.4×10^{-11}	44–89[b]	Mouse plantar nerve–lumbrical muscle	(85)
Pure type E neurotoxin[d]	1×10^{-11}–1×10^{-13}	80–320[a]	Mouse phrenic nerve–hemidiaphragm	(25)
Pure type E neurotoxin[d]	1×10^{-11}	75 ± 7[a]	Mouse phrenic nerve–hemidiaphragm	(25)
Pure type E neurotoxin[d]	1×10^{-11}	104 ± 10[a]	Rat phrenic nerve–hemidiaphragm	(25)
Pure type E neurotoxin[d]	1×10^{-11}	97 ± 10[a]	Hamster phrenic nerve–hemidiaphragm	(25)
Pure type E neurotoxin[d]	1×10^{-11}	101 ± 8[a]	Guinea pig phrenic nerve–hemidiaphragm	(25)

[a]Time to reach 90% paralysis.
[b]Time to reach complete paralysis.
[c]Time to reach 50% paralysis.
[d]In all cases type E is dichain (after trypsinization).

in a dose- and time-dependent fashion within a range of 1–100 nM type A NT and 0.5–100 nM type E NT concentrations.

Looking Ahead

A recent opinion (19) considering the production and purification of botulinum toxins for clinical use, in accordance with appropriate standards of quality, safety, and good manufacturing practice, is notable: "These restrictions required culturing in simplified medium without the use of animal meat products and purification by procedures not involving synthetic solvents or resins [and] avoided exposures to substances such as added enzymes or columns of synthetic resins, used in some methods, that could contaminate the preparation and be carried into the final injected preparations . . . We do not recommend the use of methods of purification involving enzymes, various exchangers, or synthetic solvents because of the chance of contamination."

This view, on the side of caution (for safety), appears to contain the following contradictions. Toxin production (68) starts with a medium containing animal meat products—beef infusion and chopped meat; before crystallization, the toxin is precipitated with ethyl alcohol, a synthetic solvent. The toxin is filtered for sterility and stored in the presence of human serum albumin (19). Chromatography at present is a highly dependable and reliable technique that yields pure products with extraordinary reproducibility. If one assumes that column resins may somehow be a possible source of contamination, the same degree of possibility should then be applicable to the filters used for sterile filtering the toxin because "something" may leach out of the filter material.

The recommended extremely conservative guidelines only perpetuate the technology of 1946 (10), slightly modified in 1957 (68), and merely discourage the use of improved-quality crystallized type A toxin (72) and investigation of the clinical use of the pure NTs (mol. wt. 150,000) and chimeric toxins, because their production will require chromatography. The recommendations from the investigators in England (92), are prudent and more forward-looking. Interestingly, the quality of the toxin prepared in England with modern protein chemistry techniques does not rely on its optical properties (see ref. 48 and 11 in 92), presumably because of the fallacies discussed earlier.

Secure information on the structure of the NTs (mol. wt. ~150,000), the biological activities of the different segments of the NT molecule, the structure-function relationship, and the mechanism of action of the NT is rapidly emerging. It is hoped that more imaginative and courageous scientists and clinicians will team up to further exploit the new information to provide supportive insight into the clinical application of the NT and refinements in the regimen and response.

NOTE ADDED IN PROOF

The neuronal proteins (and some of their cleavage sites) proteolytically cleaved by botulinum neurotoxin serotypes A, C, D, E, and F, which were identified after this chapter was written, are as follows: neurotoxin serotypes A and E cut SNAP25 (soluble NSF attachment protein of mol. wt. 25,000), serotype C cuts syntaxin, serotypes D and F cut VAMP isoform 2. Unlike serotype B, which cuts VAMP between Gln 76–Phe 77, type D cuts VAMP between Ala 67–Asp 68 as well as between Lsy 59–Leu 60, and type F cuts VAMP between Gln 58–Lys 59. In contrast to VAMP (vesicle-associated membrane protein), syntaxin is a protein embedded on the acceptor membrane and SNAP is cytoplasmic protein that associates transiently with membranes (93–95). A diagram on p. 488 of Ref. 95 illustrates the relative positions of these proteins involved in docking of the vesicle with presynaptic membrane.

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